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Species D Adenoviruses as Oncolytics against B-cell Cancers

Christopher Y. Chen¹, Julien S. Senac², Eric A. Weaver¹, Shannon M. May¹, Diane F. Jelinek^{3,4}, Philip Greipp^{4,5}, Thomas Witzig^{4,5}, and Michael A. Barry^{1,3,6}

Abstract

Purpose: Oncolytic viruses are self-amplifying anticancer agents that make use of the natural ability of viruses to kill cells. Adenovirus serotype 5 (Ad5) has been extensively tested against solid cancers, but less so against B-cell cancers because these cells do not generally express the coxsackie and adenoviral receptor (CAR). To determine whether other adenoviruses might have better potency, we "mined" the adenovirus virome of 55 serotypes for viruses that could kill B-cell cancers.

Experimental Design: Fifteen adenoviruses selected to represent Ad species B, C, D, E, and F were tested *in vitro* against cell lines and primary patient B-cell cancers for their ability to infect, replicate in, and kill these cells. Select viruses were also tested against B-cell cancer xenografts in immunodeficient mice.

Results: Species D adenoviruses mediated most robust killing against a range of B-cell cancer cell lines, against primary patient marginal zone lymphoma cells, and against primary patient CD138+ myeloma cells *in vitro*. When injected into xenografts *in vivo*, single treatment with select species D viruses Ad26 and Ad45 delayed lymphoma growth.

Conclusions: Relatively unstudied species D adenoviruses have a unique ability to infect and replicate in B-cell cancers as compared with other adenovirus species. These data suggest these viruses have unique biology in B cells and support translation of novel species D adenoviruses as oncolytics against B-cell cancers. *Clin Cancer Res*; 17(21); 6712–22. ©2011 AACR.

Introduction

B-cell malignancies can present as leukemias, myelomas, and lymphomas. In the United States, there are more than 300,000 patients afflicted by these diseases for which chemotherapy, radiation therapy, and stem cell transplantation are the main treatments (1, 2). The spectrum of B-cell cancers are thought to arise at different stages of the ontogeny of normal B cells. For example, chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) may arise from intermediate and mature B cells; non-Hodgkin's lymphomas are thought to be generated from mature immunoblasts; and myeloma is thought to arise

from differentiated plasma cells. Although therapies have improved survival in many of these cancers, 5-year relative survival is still only 60% for lymphomas, 50% for leukemias, and 35% for myeloma. Therefore, complementary therapies are needed to address those cancers that resist current treatments.

Recent work has explored the use of viruses as cancer therapies (reviewed in ref. 3). Certain viruses have cancer killing or "oncolytic" capacity based on their intrinsic ability to kill cells. These oncolytic viruses have appeal because they are "self-amplifying" drugs, because each infected and killed cancer cell can produce thousands of progeny virions that can kill other cancer cells.

Adenoviruses (Ad) are nonenveloped DNA viruses that are among those being tested as oncolytic agents (3, 4). There are currently 55 serotypes of Ad that infect humans and these serotypes distribute into 7 species (A–G). The vast majority of oncolytic testing has been focused on 1 virus, Ad serotype 5 (Ad5) from species C. Ad5 has been extensively tested for killing solid epithelial tumors in part due to its ability to infect these cells via the coxsackie and adenovirus receptor (CAR; reviewed in ref. 4). Although Ad5 can be quite potent particularly against epithelial solid tumors, 27% to 100% of humans are already immune to this virus. Therefore, its utility may be restricted as an oncolytic treatment for many patients.

Ad5 is markedly less potent against cells that do not express CAR including most of the cells of the hematologic system (5, 6). For this reason, Ad5 has not been applied

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Translational Relevance

This work analyzes the potential use of novel adenovirus serotypes as therapeutic agents against B-cell cancers. This work was done in part using primary patient samples from myeloma and lymphoma patients to ensure that the observations are relevant to clinical translation. This work lays the foundation for generating conditionally replicating adenoviruses engineered for selective systemic therapy against B-cell cancers in human patients.

extensively as an oncolytic against hematologic cancers. This ineffectiveness is curious, particularly in light of the fact that Ad5 and other Ad serotypes are frequently isolated from human lymphoid tissues and peripheral blood (reviewed in ref. 7). For example, species B, C, and E Ads have been isolated or detected in human adenoids, tonsils, and peripheral blood mononuclear cells (PBMC). Consistent with this, species B viruses like Ad35 that bind CD46 are substantially better at infecting cells of hematologic origin (5, 6). Therefore, it is possible that other adenoviral serotypes may be better than Ad5 as oncolytics against hematologic malignancies.

Given there are 54 other serotypes of adenoviruses in 7 biologically diverse species, we recently screened low seroprevalence Ads from species B, C, D, and F for oncolytic activity against the B-cell cancer myeloma (8). In this work, we found that 2 species D viruses, Ad26 and 48, were more potent than all other viruses including species C Ad5. This was notable, because species D viruses make up 32 of the 55 known human Ads. Despite their abundance, this large and diverse group of viruses is also the least studied for basic virus biology and as oncolytics.

Given this and their high activity against B-cell-derived myeloma cancers, we hypothesized that species D adenoviruses might have particular efficacy against B-cell cancers, and that viral oncolysis might vary with the maturation state of the B-cell cancer cells. To test these hypotheses, 7 species D adenoviruses were compared with 6 viruses from species B, C, and E for their ability to kill B-cell cancers that arose from various stages of B-cell ontogeny including CLL, different stages of B-cell lymphoma, and myeloma.

Materials and Methods

Cell lines

The mouse myeloma cell line 5TGM1 and human Burkitt's lymphoma line Raji were generously provided by Dr. Stephen Russell (Mayo Clinic). CHO-CD46 expressing cells were generously provided by Dr. Kah Whye Peng (Mayo Clinic). Human lymphoma RL and Hep3B human hepatocarcinoma cells were obtained from American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 supplemented with 10% FBS (HyClone). The human cancer cell lines Mec-1 and Mec-2 grew spontaneously from a patient with B-CLL (9). Mec-1 and 2 cells were main-

tained in Iscove's modified Dulbecco's medium (IMDM) + Glutamax supplemented with 10% FBS. Human cancer cell line A549 (lung carcinoma) from ATCC was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Patient samples

PBMCs, CLL, lymphoma, multiple myeloma, and reactive follicular hyperplasia cell samples were collected at the Mayo Clinic from patients after informed consent and Institutional Review Board approval had been obtained. Mononuclear cells were isolated from postapheresis leukocyte reduction chambers from healthy blood donors as described (10). Monocyte populations were isolated using CD14+ immunomagnetic bead selection followed by sequential isolation of CD3+ cells immunomagnetic selection for T cells (both from Miltenyi Biotec). Labeled cells were separated on an AutoMACS separator (Miltenyi Biotec) running the POSSEL program. Remaining cell populations after sequential isolation of monocyte and T cells were lymphoid cells that are predominantly B cells. Typical isolation purities for the positive selected cells are greater than 95% (10). Patient cells were maintained in culture in IMDM supplemented with 10% FBS.

Viruses

Ad4, Ad5, Ad6, Ad7, Ad11, Ad17, Ad24, Ad26, Ad28, Ad30, Ad35, Ad45, and Ad48 were obtained from the ATCC. Viruses were propagated in HEK 293 cells (also from ATCC). Viruses were purified by CsCl purification and quantitated by determining the optical density at 260 nm (OD₂₆₀).

Analysis of *in vitro* infection and killing by Trypan Blue uptake

Cell lines and/or patient cells were infected with 10,000 virus particles (vp) per cell for 1 hour at 4°C. Cells were then washed 3 times with Hank's buffered saline solution (HBSS, Invitrogen) and placed in a 37°C incubator for the indicated length of time. Loss of membrane integrity was assessed by Trypan Blue uptake as described by Barry and colleagues (1990). For neuraminidase-treated samples, cells were incubated with 5 µg/mL neuraminidase (SIGMA) at 4°C for 1 hour prior to incubation with virus.

Quantitative real-time PCR

Virally infected cells were harvested at the indicated time points and DNA was isolated using DNeasy kit from Qiagen. 100 ng of DNA was used in a real-time PCR assay using SybrGreen reagent to quantitate viral genomes in each sample. Samples were read using an ABI Prism 7900HT real-time instrument. A standard curve of the corresponding viral plasmid DNA was used for quantitation, using primers designed against hexon. Measurements were made in triplicate.

Cell viability MTT assay

To assess blocking of viral binding, 4×10^4 Hep3B cells were plated into each well of 96-well plates. Cells were then incubated in HBSS containing 500 μ L of 200 μ g/mL lectin from wheat germ agglutinin (SIGMA), 200 μ g/mL mannose (SIGMA), or 22 mg/mL of cyclic 4-RGD peptide (Invitrogen) for 1 hour at 4°C. A total of 4×10^7 of the indicated viral particles were then added to each well. After 7 days, cell viability was assessed by MTT assay (SIGMA). $n = 3$.

Blocking receptor binding

A total of 5×10^5 Hep3B cells were plated in each well of 24-well plates. An amount of 100 μ L of 200 μ g/mL lectin from wheat germ agglutinin, 200 μ g/mL mannose, or 22 mg/mL of cyclic 4-RGD peptide were added and incubated with cells for 1 hour at 4°C. A total of 5×10^8 particles of Ad26 were added to each well and left to incubate for 1 hour at 37°C. Wells were then washed 3 times with HBSS. DNA was isolated from the cells using DNeasy from Qiagen and quantitated by real-time quantitative PCR (qPCR). $n = 3$.

Ad26 CD46 receptor blocking

A total of 4×10^4 human reactive follicular hyperplasia patient sample cells or Hep3B cells were plated into 96-well plates. Cells were then incubated with 1.5 μ g of IgG1 mouse isotype antibody (BD Pharmingen), mouse anti-human CD46 from BD Pharmingen (anti-CD46a) or mouse anti-human CD46 antibody from AbD Serotec (CD46b) dialyzed in HBSS were added to each well. Plates were then incubated at 4°C for 1 hour. A total of 4×10^7 of the indicated viral particles were then added to each well. After 7 days, cell viability was assessed by MTT assay or crystal violet staining. $n = 3$.

Human lymphoma xenografts

A total of 3×10^6 RL (human lymphoma) cells were injected subcutaneously into the hind flank of 4- to 6-week-old nude mice (Harlan Sprague-Dawley). After palpable tumors formed (14 days), the mice were injected intratumorally with 3×10^{10} viral particles of wild-type virus in 100 μ L total volume of HBSS. Control groups were injected with buffer alone. Tumors were observed every other day and measured weekly. Volume was calculated as $\frac{1}{2}$ length \times width \times width. When animals were found with tumor volumes exceeding 2,000 μ L, ulcerated tumors, or greater than 20% weight loss, they were sacrificed. $n = 6$.

Animals

All animal experiments were carried out according to the provisions of the Animal Welfare Act, Public Health Service Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Mayo Clinic. Female nude mice (4–6 weeks old) were purchased from Harlan.

Statistics

P values represent 2-tailed *t* test values unless otherwise indicated. $P < 0.05$ considered significant.

Results

Infection and killing of PBMCs by adenoviruses

Adenoviruses from species B, C, and E have frequently been identified in normal human PBMC and lymphoid tissues (7). Our previous testing of adenoviruses from species B, C, and D against bone marrow cells from myeloma patients found that species D Ads 26 and 48 were most potent at killing myeloma cells while largely sparing normal marrow cells (8). These data suggest that different Ad serotypes from different virus species may have unappreciated tropism for human hematologic cells.

To explore the ability of different Ads to infect and kill normal cells, PBMCs from healthy volunteers were enriched for CD14⁺ monocytes and CD3⁺ T cells by positive selection. The remaining cell population after sequential isolation of monocyte and T cells was predominantly (95%) B cells (10). These cells were then incubated with adenovirus representatives from different species. Ad serotype 5 (Ad5) was tested as a benchmark oncolytic because it is the only virus used to date in humans for virotherapy. Other adenoviruses were chosen based on the following criteria. First, their viral infection is well tolerated by humans. Viruses associated with cancer (species A) or obesity (species D Ad36) were excluded. Previously tested species F gastrointestinal viruses Ad40 and Ad41 were omitted as these were ineffective in all tests (Ref. 8; and data not shown). Second, new viruses that were tested here had to have low seroprevalence in humans to avoid neutralization in patients if they were translated into the clinic. Third, the viruses were genetically distinct and more likely to possess novel phenotypes that might be more effective against B-cell cancers.

Each cell population was incubated with each of the Ads and cell viability was assessed by Trypan Blue exclusion more than 7 days (Fig. 1). Addition of most of the viruses to CD14⁺ monocytes reduced viability by 20% to 45% as

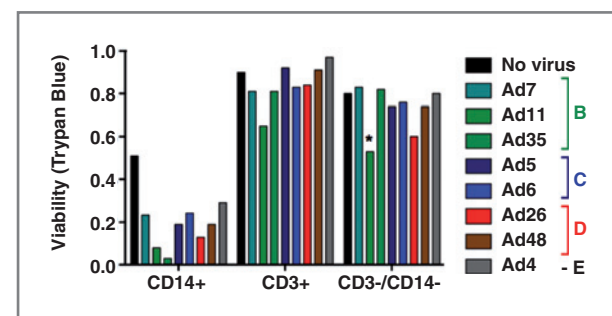


Figure 1. Comparison of adenoviral serotypes for infection and killing of normal PBMCs. The indicated cells were incubated for 7 days with the indicated wild-type adenoviruses and cell viability was assessed by Trypan Blue exclusion. MOI = 10,000 viral particles/cell.

compared with controls. However, the untreated CD14⁺ monocytes lost 50% viability, so toxicity by the viruses was likely exaggerated by the nonoptimal culture conditions. Treatment of CD3⁺ T cells with most of the Ads caused little loss of viability. The one exception was Ad11 that reduced survival approximately 25%. Most of the viruses had little effect on CD3⁺/CD14⁺ cells with the exception of Ad11 that reduced viability 30% ($P = 0.0309$). Ad26 also seemed to have some toxicity to these cells; however, this did not reach statistical significance. These data suggest that normal T and B cells are relatively resistant to infection by most Ad species, but that there is some evidence of cell killing by select viruses. This is consistent with previous tests in primary marrow cells (8). This is also consistent with observations of persistent association of Ads with lymphocytes (11).

Oncolytic screen of Ad species on CLL and lymphoma cell lines

Our previous data suggested that species D viruses were most potent at killing primary patient myeloma cells while largely sparing normal cells (8). Myeloma arises from mature B-cell plasma cells, the last step of B-cell maturation. Given their activity against these mature B-cell cancers, we tested select adenoviruses against B-cell cancers arising from various stages of B-cell maturation. These included CLL and B-cell lymphomas arising from various stages of B-cell ontogeny.

Two viruses from each of species B, C, and D were first tested on B-cell cancer cell lines representing cancers arising at different stages of B-cell development: MEC1, MEC2, Raji, and RL cells. MEC1 and MEC2 cells are human CLL cell lines that express mature B-cell markers CD19, CD20, CD21, and CD22. They are CD46 positive and coxsackie and adenovirus receptor (CAR) negative. These 2 CLL cell lines were derived from the same patient and differ mainly in the expression of CD23 and FMC7 (9). RL cells are human lymphoma cells that are EBV negative. RL cells are derived from the ascites of a patient with diffuse large cell lymphoma (12). Raji cells are an EBV-positive lymphoma cell line derived from a patient with Burkitt's Lymphoma (13). Myeloma cell lines were tested previously (8).

Species B Ad11 and 35, species C Ad5 and 6, and species D Ad26 and 48 were incubated with the indicated cell lines and cell killing was monitored over time by loss of membrane integrity assessed by Trypan Blue uptake (Fig. 2A–D). Under these conditions, species B viruses were largely ineffective against the CLL or lymphoma cell lines. Species C Ad5 and Ad6 mediated intermediate killing over time on all 4 cell lines, with Ad5 being slightly more effective than Ad6. Consistent with results on myeloma, species D viruses Ad26 and Ad48 were most effective in killing all of these B-cell cancer cell lines with Ad26 generally being most effective.

Replication of viruses in lymphoma cell lines

In most cases, oncolysis depends on the virus proceeding through its life cycle. To test whether oncolysis of these cell

lines correlated with propagation of the virus life cycle, adenoviral genome replication was assessed by real-time qPCR at varied times after incubation with Epstein-Barr Virus (EBV) positive Raji lymphoma cells and with EBV-negative RL lymphoma cells. In addition to monitoring viral genomes associated with the cells, qPCR after 1 hour incubation at 4°C with the cells also provides a relative assessment of the number of virions that have successfully bound to the cells.

qPCR testing of each of the viruses incubated for 1 hour with Raji and RL cells revealed that the species B viruses Ad11 and Ad35 bound most effectively (Fig. 2E and F). Although these viruses were most effective at binding, they failed to replicate their DNA. This is consistent with both their inability to kill the cells and with previous observations on primary patient myeloma cells (8). In contrast, the species C and D viruses bound to lower degrees, but replicated their genomes and killed the cells. This higher replication and killing of cells by species C and D viruses is consistent with previous data in more mature myeloma cancer cells (8).

Oncolytic testing on primary patient CLL and lymphomas

While testing in cell lines provides useful guidance of potential therapy, there is no substitute for testing in primary patient cancers. To test this, CLL samples were collected from 2 patients and these were incubated with Ad5, 6, 11, 35, 26, and 48 (Fig. 3A and B). In this case, cell killing was at most 50% greater by species D viruses than the other viruses after 7 days. This is markedly weaker than in immortalized CLL cell lines. Again, the species D viruses Ad26 and Ad48 mediated the highest level of killing on these B-cell cancers.

Patient samples from 2 grades of lymphoma were tested with species B Ad7, Ad11 and Ad35, species C Ad5 and Ad6, species D Ad26 and Ad48, and also with species E Ad4. When tested on follicular lymphoma, the viruses only mediated 35% cell killing within 7 days (Fig. 3C). Interestingly, the viruses that utilize CAR (Ad4, 5, and 6) were most potent against this particular type of solid tumor lymphoma. In contrast, when tested against diffuse large B lymphoma, many of the viruses had similar oncolytic activity with the most potent virus being Ad26 (Fig. 3D).

Expanded screen of species D adenoviruses

These observations in CLL, lymphoma, and myeloma indicated that the 2 species D viruses, Ad26 and Ad48, were generally most effective against cancers spanning B-cell development. This observation is interesting because these viruses are largely unstudied despite constituting more than half of the 55 human Ads.

On the basis of this, we hypothesized: (i) that species D viruses might have a unique tropism for B-cell cancer cells; (ii) that this tropism might make them particularly useful as oncolytics in patients; and (iii) that species D viruses might have differing abilities to kill B-cell cancers from different steps of B-cell ontogeny.

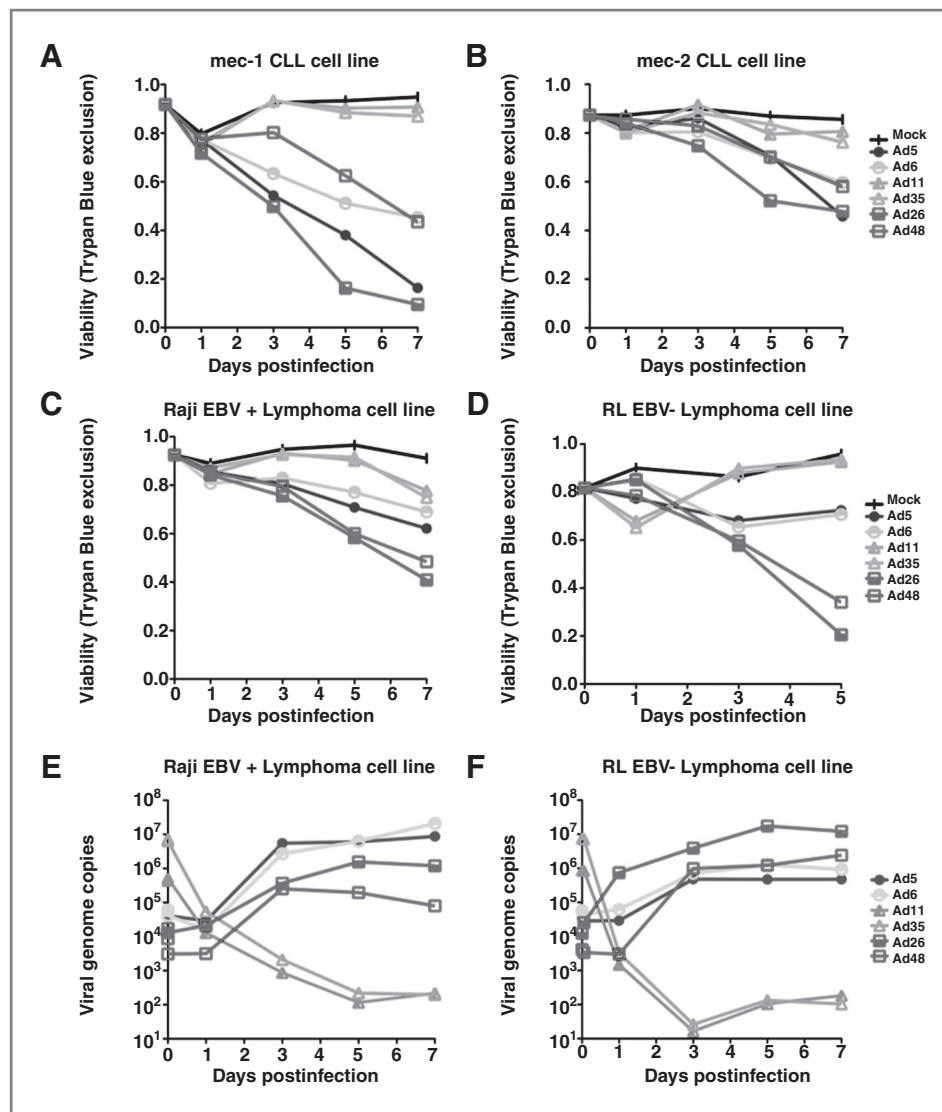


Figure 2. Comparison of adenoviral serotypes for infection and replication in B-cell cancer cell lines. Comparison of cell killing of either MEC1 (A) or MEC2 (B) CLL cell lines by adenoviruses as assessed by Trypan Blue uptake following infection with virus at an MOI of 10,000 viral particles/cell. Killing of either Raji (C) or RL (D) human lymphoma cell lines by adenoviruses as assessed by Trypan Blue uptake. E and F, real-time qPCR showing viral genome copies present within cells more than 1-week time course following infection for cells in C and D. $n = 3$.

To test these hypotheses, a broader set of species D viruses including Ads 17, 24, 26, 28, 30, 45, and 48 were compared with species B Ads 7, 11, and 35, species C Ads 5 and 6, and species E Ad4. In agreement with results obtained using the smaller panel of viruses (Fig. 2), all species D viruses were superior to species B, C, and E viruses for killing the RL EBV-negative lymphoma cells (Fig. 4A). Species C and E viruses mediated intermediate levels of cell killing. Species B viruses again showed no significant killing effect.

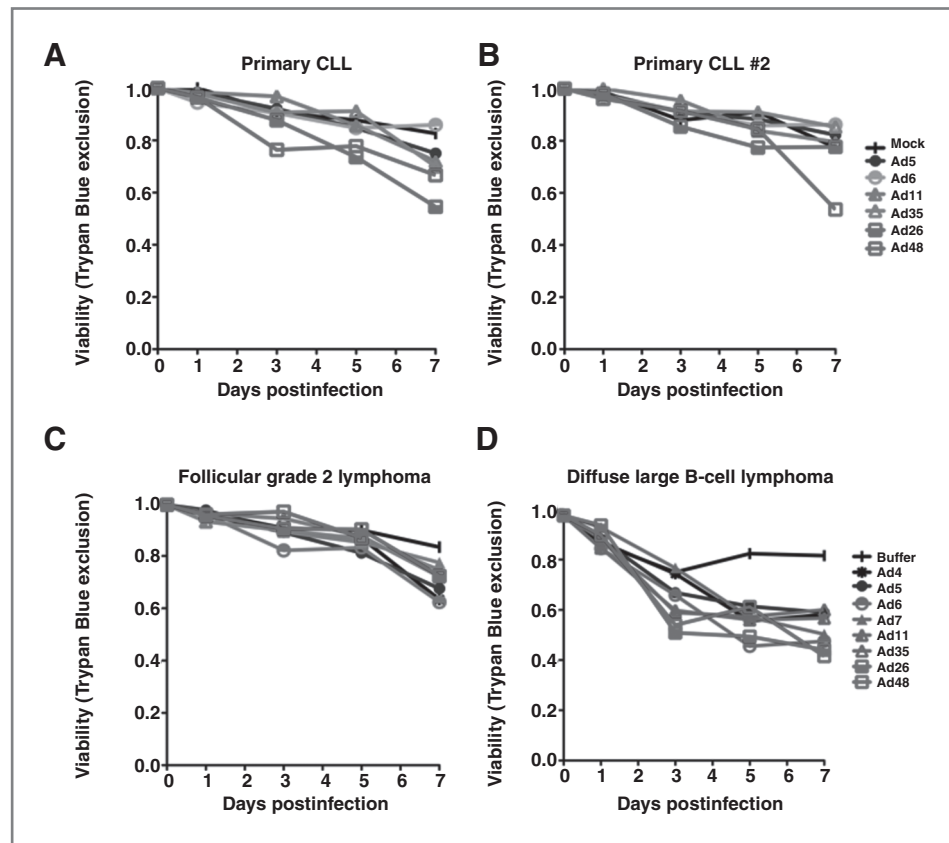
When the 13 virus serotype panel of viruses was tested against primary marginal zone lymphoma cells from a patient, these cells were efficiently killed by all of the species D viruses. No other viruses were effective against these cells.

The 13 virus serotype panel was also tested on cells from a multiple myeloma patient. Testing on bone marrow samples from myeloma afforded the opportunity to com-

pare viral cell killing on normal cells versus cancer cells because these cells can be separated by purification on CD138 antibody beads. When the 13 virus panel was tested on CD138-negative (Fig. 4C) and CD138-positive marrow cells, the species D viruses were the most effective at killing the CD138+ malignant cells (Fig. 4D). In contrast, markedly lower cell killing was observed in CD138-noncancerous cells.

Due to the limited availability of patient sample cells and the large number of viruses being screened in the Trypan Blue viability assays, Figs. 2–4 were each tested with only 1 sample. To compare the oncolytic activity of the viruses against the highly proliferative patient B-cell cancers statistically, data from the marginal zone lymphoma patient was pooled with that from multiple myeloma patients from Figs. 4 and 7 as well as 2 additional multiple myeloma patient samples not included within the figures presented (Fig. 5). Although species B, C, or E viruses showed no

Figure 3. Comparison of adenoviral serotypes for infection and replication of primary B-cell cancer cells. A and B, 1 week cell viability of 2 different CLL patient's sample cells after infection with 6 different adenoviral serotypes. C, viability of follicular grade 2 cancer lymphoma patient sample cells following infection by a panel of 8 adenoviral serotypes. D, viability of diffuse large B-cell lymphoma patient sample cells following infection by panel of 8 adenoviral serotypes. Experiments were done using a viral MOI of 10,000 particles/cell.



consistent effect on the 5 patient samples, all of the species D viruses showed significant cell killing (Ad17, $P = 0.0031$; Ad24, $P = 0.0002$; Ad26, $P < 0.0001$; Ad28, $P < 0.0001$; Ad30, $P = 0.0004$; Ad45, $P < 0.0001$; Ad48, $P = 0.0003$). Given the inherent heterogeneity between these patient samples, the higher activities of the species D viruses seem significant.

***In vivo* testing of Ads in mouse xenografts of human lymphoma**

To evaluate whether *in vitro* studies could be relevant to *in vivo* therapy, RL lymphoma tumors were initiated by subcutaneous injection of the cells into the flanks of nude mice. Once tumors reached volumes of 200 mm³, groups of 6 mice were injected intratumorally a single time with 3×10^{10} vp of selected viruses. Because species D Ad26 and Ad45 were generally the most potent of the 13 tested Ads, these 2 viruses were tested. For comparison, another group of mice were injected with the same dose of Ad5 (species C) that was less effective *in vitro*. A final group was injected with buffer alone. Tumor volume was measured weekly (Fig. 6). Under these conditions, both Ad26 and Ad45 mediated significant delay in tumor growth after only a single injection. In contrast, both buffer-treated and Ad5-treated tumors grew unchecked requiring animals to be sacrificed within 2 weeks.

Receptor utilization by species D adenoviruses

These data indicate that species D viruses have unique efficacy against B-cell cancers spanning the ontogeny of normal B cells. One of the main differences between the different subgroups of adenovirus is their receptor specificity. Species C and E Ads are known to bind cellular CAR with high affinity and utilize their fiber proteins to then bind and enter cells via interactions of penton base with α_v integrins (14, 15). In contrast, species B viruses bind CD46 and perhaps other receptors with their fibers (16–18).

Despite the fact that species D Ads represent more than half of all human adenoviruses, these viruses are diverse and relatively unstudied. Species D viruses can bind CAR, CD46, and sialic acid with their fiber proteins (Refs. 19–23 and Supplementary Fig. S1). Although they can bind all of these receptors, species D Ads have short fibers with only 8 repeats making them unable to efficiently enter cells using CAR (21). When Ad26 and 48 were vectored as replication-defective vaccines, receptor utilization studies suggested that they did not use CAR and only weakly used CD46 as a receptor (24). Sialic acid binding was not tested. From this, it is unclear whether all species D viruses use the same receptors and if receptor specificity of these viruses explains their unique ability to kill B-cell cancers.

These data suggested that the subgroup D viruses might use sialic acid, CAR, or CD46 for binding and killing B-cell

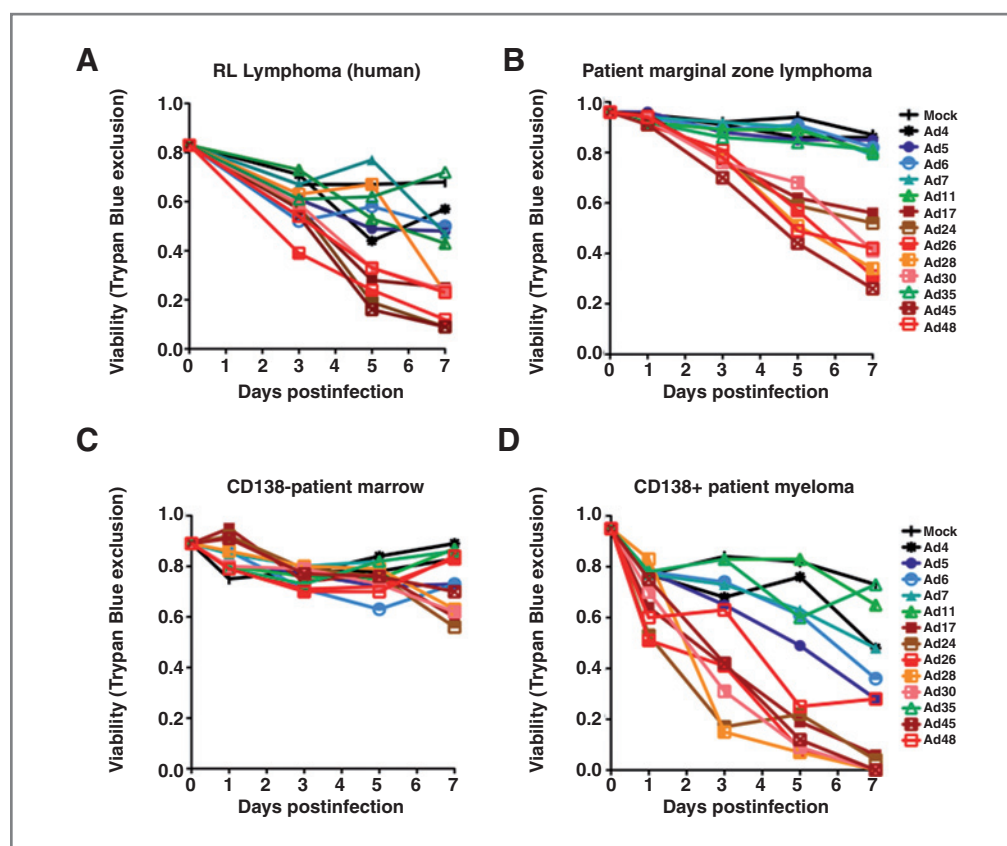


Figure 4. Expanded comparison of adenoviral serotypes for infection and replication in B-cell cancer cells. A 13-member panel of adenoviral serotypes are tested against 2 B-cell cancers. Lymphoma: A, RL human lymphoma; B, patient marginal zone lymphoma. Myeloma: C, nonmalignant CD138– patient bone marrow cells; D, malignant CD138+ patient bone marrow cells. Viability was assessed by Trypan Blue uptake following infection at an MOI of 10,000 viral particles/cell. Green, species B; blue, species C; yellow, orange, red, species D; and brown, species E.

cancers. An alternate possibility is that the viruses may be binding to α v integrins with RGD motifs on penton base (19). To test these possibilities, virus infection was assessed with and without incubation with agents that could block interactions with these receptors (Fig. 7). Because a sialic acid binding motif of fiber is well conserved on these species D viruses (Supplementary Fig. S1), they were tested for their ability to bind sialic acid by treating primary CD138+ myeloma cells with neuraminidase to remove cell surface sialic acid residues prior to incubation with the viruses (Fig. 7A). Under these conditions, no obvious effects of removal of sialic acid were observed. To test the role of integrin binding by RGD motifs on the viruses, Hep3B cells were incubated with the cyclic RGD-4C peptide (25) prior to virus exposure and cell viability was measured 7 days later (Fig. 7B). In contrast to the weak effects of neuraminidase, the RGD-4C peptide partially inhibited oncolysis by species D viruses Ad17, 24, 26, and 48 ($P = 0.0076$, $P = 0.0019$, $P = 0.0011$, and $P = 0.0091$, respectively), but not cell killing by species D viruses Ad28, 30, or 45 ($P = 0.8717$, $P = 0.8914$, and $P = 0.0609$, respectively). To test this further, Hep3B cells were incubated with wheat germ agglutinin (WGA) to

block cell surface sialic acids or with cyclic RGD before addition of the potent Ad26 virus (Fig. 7C). qPCR for viral genomes showed no reduction in virus binding by WGA. In contrast, cRGD inhibited Ad26 binding more than 50% ($P = 0.034$). To test whether CD46 binding was involved in virus killing, primary patient reactive follicular hyperplasia cells were incubated with a blocking CD46 antibody or with isotype control antibody before the addition of species B Ad7, species C Ad5, and species D Ad26. Consistent with previous results, Ad26 mediated more cell killing than Ad5 or Ad7. Incubation with the CD46 antibody reduced cell killing nearly 50% ($P = 0.067$). Combination of CD46 antibody and cyclic-RGD peptide on patient myeloma cells mediated complete protection against killing by Ad26, suggesting that both receptors are being utilized by the virus (Supplementary Fig. S2).

Discussion

Following the first government approval for the use of oncolytic adenovirus in the treatment of head and neck cancers, researchers have been exploring the use of replication competent virus in other cancer treatments (3, 26).

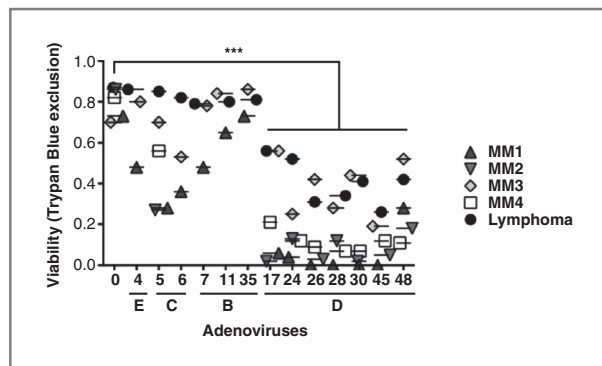


Figure 5. Pooled cell viability data from 5 patient B-cell cancers. The marginal zone lymphoma patient sample data was pooled with cell killing data of 4 patients' multiple myeloma samples. Individual viabilities (y-axis) from each experiment are presented as points above the viral serotype tested (x-axis). ***, $P < 0.001$.

Ad5 is the most common vector being studied for this purpose. However, 27% to 100% of people in populations throughout the world are predicted to have neutralizing antibodies against Ad5 viral proteins (24, 27). The presumed neutralization of the therapeutic vector in a large group of individuals makes Ad5 a poor choice for an oncolytic in these patients. More recent studies have probed alternative less prevalent adenoviruses for efficacy against cancer cells (8, 28–32). In a recent study against myeloma, Ad5 and Ad6 species C viruses showed moderate anticancer effect against cell lines and primary multiple myeloma patient cells. However, Ad26 and Ad48 species D viruses were more proliferative in these cells (8). This is surprising considering species D viruses do not infect human solid tumor cancer cell lines (33). On the basis of these data, we hypothesized that uncommon species D viruses are superior to Ad5 for the treatment of B-cell cancers.

To test this hypothesis, species B, C, and D adenoviruses were tested against B lineage cancer cell lines and primary

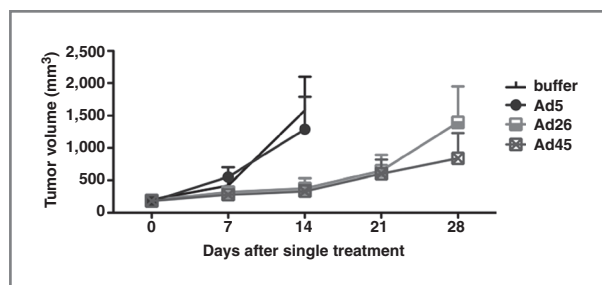


Figure 6. *In vivo* comparison of select adenoviral serotypes for killing of lymphoma xenografts in nude mice. RL lymphoma tumors were initiated subcutaneously in nude mice. Groups of 6 tumor-bearing mice were injected intratumorally a single time with 3×10^{10} vp of the indicated adenoviruses. Tumor size was monitored over time after injection and mean tumor sizes are shown. Lines end when the first animal of the group had to be sacrificed to avoid misleading skewing of the lines as larger tumors are removed.

patient samples. As predicted by our previous myeloma study, Ad26 and Ad48 and all tested species D viruses were effective against all of the cell lines. When the viruses were tested against primary patient CLL, myeloma, and lymphoma samples, the species D viruses were again most effective, but were not as active against CLL as against lymphoma and myeloma cells. Given that immortalized cell lines acquire additional modifications, it is not entirely surprising that a dichotomy in responses was observed between CLL cell lines and primary samples. Fortunately, the species D viruses remained active against most of the primary lymphoma samples and against all tested myeloma cancer cells. In future studies, it may be interesting to further characterize cells that are refractory to cell killing. For example, it would be useful to determine the precise changes in cellular receptor expression that occur as primary cells are converted to immortalized cell lines. In primary patient sample cells, refractory populations are likely nonmalignant. Better sorting methods may reduce the number of these cells within samples.

These data suggest that species D adenoviruses are most effective against lymphomas and myelomas—the B-cell cancers arising from the later stages of B-cell ontogeny. To some degree, there seems to be correlation between the ability of the viruses to kill B-cell cancers and their proliferative capacity. This is particularly notable in the ability of the viruses to kill immortalized CLL cell lines, but to have markedly less effect on primary CLL samples and slower growing lymphomas.

Slow growing B-cell lymphomas and leukemias include: follicular lymphoma (the most common slow-growing NHL), CLL (also known as SLL), lymphoplasmacytic lymphoma, marginal zone lymphoma, mucosa-associated lymphoid tissue (MALT) lymphoma, and Waldenström macroglobulinemia. Fast growing NHLs include diffuse large B-cell lymphoma (the most common fast-growing NHL), AIDS-associated lymphoma, anaplastic large-cell lymphoma, Burkitt lymphoma, central nervous system lymphoma, lymphoblastic lymphoma, MALT lymphoma, mantle cell lymphoma, and peripheral T-cell lymphoma.

The CLL samples, and 2 of the 3 lymphoma samples obtained for this study were follicular grade 2 lymphoma and marginal zone lymphoma in the slow growing lymphoma group. The third lymphoma sample was a diffuse large lymphoma in the fast-growing group. Although the species D adenoviruses killed all of these cancers, there was no clear correlation between general cancer growth phenotype and oncolytic susceptibility. This could reflect the artificial *in vitro* testing that does not necessarily recapitulate the growth patterns of these cells *in vivo*. Future clinical testing will be needed to determine whether an association with growth and oncolysis exists.

Because species D viruses were selective against lymphoma cell lines and patient cells, the 2 best conducting species D viruses Ad26 and Ad45 were compared with Ad5 for *in vivo* efficacy against RL tumor xenografts. In this case, a single intratumoral injection of 3×10^{10} viral particles significantly slowed tumor growth and improved survival

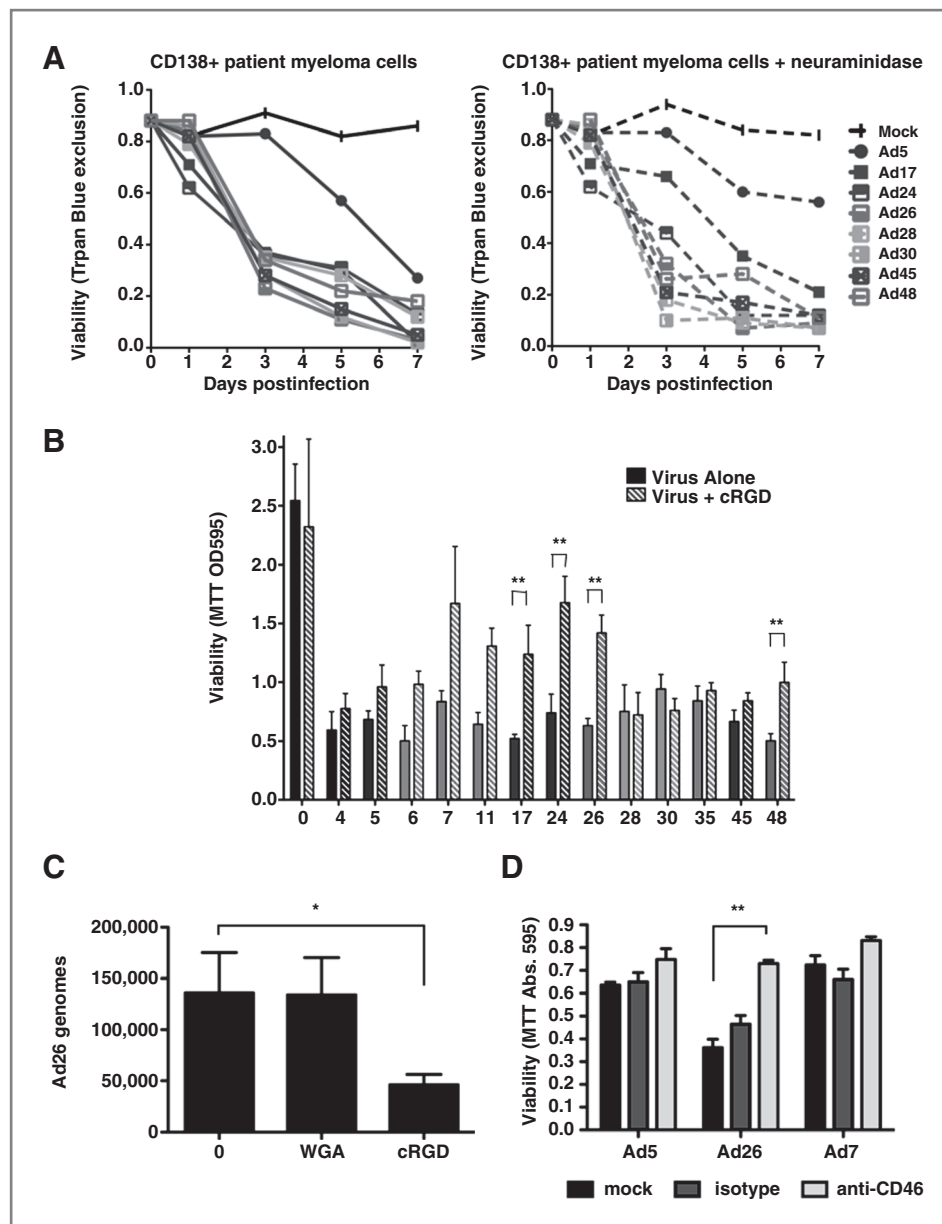


Figure 7. Receptor blocking during species D adenovirus infection. **A**, 13-panel adenoviral infection (MOI = 1,000 viral particles/cell) of patient CD138+ multiple myeloma cells with (right) and without (left) prior treatment with neuraminidase to remove sialic acid. **B**, viability of Hep3B cells 1 week following infection by the 13 adenoviruses (x-axis) in the presence or absence of cyclic RGD peptide. 0, uninfected. **C**, effects of WGA or cyclic RGD on Ad26 binding to Hep3B cells as assessed by qPCR. 0, uninfected. **D**, blocking CD46 binding of Ad26 to human patient reactive follicular hyperplasia cells by addition of isotype or anti-CD46 antibody. *, $P < 0.05$. **, $P < 0.01$.

of these animals. Ad5 was ineffective against these tumors. Although single intratumoral injection of species D adenoviruses was unable to completely kill tumors, future studies using sequential treatment injections are likely to show additional therapeutic benefit. It will be interesting to assess the response of regressive tumors to these secondary treatments. If tumor cells that are resistant to infection and killing by one species D serotype persist and clonally expand, a serotype switch approach may show the greatest antitumor effect.

These data suggest that subgroup D viruses may have utility as oncolytics against myeloma and lymphoma. Moreover, species D viruses are the only fully replication

competent adenoviruses to show efficacy against lymphoma. It should be noted that a conditionally replicating Ad5 carrying the melanoma differentiation-associated gene-7/interleukin-24 transgene was effective against a rare CAR expressing lymphoma (34). Subgroup C viruses also seem effective against myeloma; however, because of the seroprevalence of Ad5, either a subgroup D virus or Ad6 would seem a better choice of vector for future studies.

The work presented herein begs the question, what cellular factors expressed by B-cells and what viral factors related to species D viruses make species D viruses more oncolytic against B-cell cancers? Our data suggest that these viruses do not use sialic acid for oncolysis, but instead use a

combination of CD46 and integrin as receptors. Neither CD46 nor integrin blockade completely inhibited species D virus cell killing. Therefore, the viruses may use both receptors in combination as suggested by the data from Supplementary Fig. S2 or may utilize an as yet uncharacterized receptor for infection of these B cells.

Testing of viruses from species B, C, D, and E on normal PBMCs and on bone marrow cells here and previously (34) suggest that these noncancerous cells are relatively resistant to lethal infection by the viruses. Previous analysis of viral binding and replication in marrow samples from myeloma patients indicated that species B Ad11 binds both "normal" CD138[−] marrow cells and cancerous CD138⁺ myeloma cells 100-fold more strongly than species C or D viruses. This is interesting given that Ad11 was almost always the only virus that showed any cytotoxicity against normal T and B cells. Although Ad11 binds marrow cells well, it does not seem to replicate indicating that at least in marrow, this is not a productive infection. Whether it or other Ad serotypes from the diverse species tested here bind and undergo abortive or productive infections in PBMCs remains to be tested. Although most of the viruses spared normal cells from killing, safe clinical translation into patients will likely require that these species D viruses be engineered conditionally replicating adenoviruses (CRAd) for these B-cell cancers. This may be achieved by mutation of E1A or B interactions with tumor suppressors or by putting early genes under control of cancer or cell-specific promoters (reviewed in ref. 3).

In summary, adenoviruses seem to interact with normal and cancerous hematologic cells. These observations have implications for interactions of these viruses with normal blood cells and for their use as oncolytic agents. In this regard, species D adenoviruses seem most robust at killing

B-cell cancers arising from later steps in B-cell ontogeny. The specificity of this oncolytic phenotype is not precisely known but is likely due to a combination of viral fiber length and available cellular receptors. Conversion of these wild-type viruses to CRAds for cancer-specific or B cell-specific replication will likely be important next steps to maximize their efficacy and safety for clinical translation into humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

C.Y. Chen designed and conducted experiments and evaluated data. J.S. Senac designed and conducted experiments and evaluated data. E.A. Weaver provided vital new reagents. S.M. May conducted experiments. D.F. Jelinek provided vital samples. P. Greipp provided vital samples. T. Witzig provided vital samples. M.A. Barry designed experiments and evaluated data.

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Supplemental Figure 1. Alignment of the Knob Domains of Fibers. The cell binding knob domains of the tested species D adenoviruses were aligned to benchmark Ads known to bind CAR (Ad5), CD46 (Ad11), and sialic acid (Ad37). Conserved amino acids involved in binding these receptors are indicated by circles (CAR), diamonds (CD46), or triangles (sialic acid) above the sequence. Solid symbols indicate near complete conservation of the given residue. Open symbols indicate partial conservation. Diamonds, circles, and triangles to the right of the alignments indicate the number of conserved residues for that line that are present in each of the species D knobs as an approximation if the virus could utilize the receptor. Presence of many of the conserved residues would support possible use of the receptor for binding (but not necessarily infection).

Supplemental Figure 2. Blocking of both CD46 and RGD interactions with Ad26. CD138+ multiple myeloma primary patient cells were incubated with Ad26 virus with or without CD46 blocking antibody and cyclic-RGD peptide. MOI = 1,000. Viability of cells was measured by MTT assay seven days post-infection.

